422 Rec'd PCT/PTO 30 MAR 2000

FORM PTO-1390 (REV. 1-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: WCM.63

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/509687

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB98/02904	29 September 1998	30 September 1997

TITLE OF INVENTION: BIOASSAY FOR THYROID STIMULATING ANTIBODIES

APPLICANT(S) FOR DO/EO/US: Marian Elizabeth LUDGATE

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau. (see PCT/IB/308).
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:

International Preliminary Examination Report.

Search Report.

Form PCT/IB/308.

Application Data Sheet.

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09/509687	PCT/GB98/02904	WCM.63

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... $ 970.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $690.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	43 - 20 =	23	x $18.00	$ 414
Independent claims	4 - 3 =	1	x $78.00	$ 78
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $260.00	$
TOTAL OF ABOVE CALCULATIONS =				$ 1,462
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28). +				$
SUBTOTAL =				$ 1,462
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$ 1,462
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$
TOTAL FEES ENCLOSED =				$ 1,462
			Amount to be refunded:	$
			charged:	$

a. [X] A check in the amount of $ 1,462 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required by 37 CFR 1.16 and 1.17, or credit any overpayment to Deposit Account No. 25-0120. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

March 30, 2000

SEND ALL CORRESPONDENCE TO:

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NAME

35,041
REGISTRATION NUMBER

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Marian Elizabeth LUDGATE

Serial No. (unknown)

Filed herewith

BIOASSAY FOR THYROID
STIMULATING ANTIBODIES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to calculation of the filing fee, please substitute Claims 1-37 as originally filed, which appear on pages 15-20, with Claims 1-43 as amended during Chapter II of the International Phase. The pages containing Claims 1-43 are marked "AMENDED SHEET" and are attached hereto. Following the insertion of Claims 1-43, please amend these claims as follows:

IN THE CLAIMS:

Claim 3, line 1, cancel "or claim 2".

Claim 5, line 1, change "any preceding claim" to --claim 1--.

Claim 6, line 1, change "any of claims 1 to 4" to --claim 1--.

Claim 7, line 1, change "any preceding claim" to --claim 1--.

Claim 10, line 1, change "any preceding claim" to --claim 1--.

Claim 11, line 1, change "any preceding claim" to --claim 1--.

Claim 12, line 1, change "any preceding claim" to --claim 1--.

Claim 13, line 1, change "any preceding claim" to --claim 1--.

Claim 14, line 1, change "any preceding claim" to --claim 1--.

Claim 17, line 1, cancel "or claim 16".

Claim 18, line 1, change "any of claims 14 to 17" to --claim 14--.

Claim 19, line 1, change "any of claims 14 to 18" to --claim 14--.

Claim 20, line 1, change "any of claims 14 to 18" to --claim 14--.

Claim 21, line 1, change "any of claims 14 to 18" to --claim 14--.

Claim 25, line 1, change "any of claims 14 to 24" to --claim 14--.

Claim 27, line 1, change "any of claims 14 to 26" to --claim 14--.

Claim 28, line 1, change "any of claims 14 to 26" to --claim 14--.

Claim 30, line 1, change "or a kit according to any preceding claim" to --according to claim 1--.

Claim 31, line 1, change "or a kit according to any preceding claim" to --according to claim 1--.

Claim 32, line 1, change "or kit according to any preceding claim" to --according to claim 1--.

Amend Claim 33 as follows:

--33. (amended) Use of a reporter construct comprising cDNA of both

(i) a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein

and

(ii) a promoter containing cAMP response elements (CREs), whereby levels of the reactant vary with induced endogenous cAMP levels, which use is in an assay method [or in the preparation of a kit], characterised in that said assay [or kit] is as defined in [any preceding] claim 30.--

Claim 35, line 1, cancel "or claim 34".

Claim 38, line 1, cancel "or claim 37".

Claim 39, line 1, change "any of claims 36 to 38" to --claim 36--.

Claim 42, line 1, cancel "or claim 41".

R E M A R K S

The above changes in the claims merely place the national phase application in the same condition as it was

during Chapter II of the international phase, with the multiple dependencies being removed.

Respectfully submitted,

YOUNG & THOMPSON

By 

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March 30, 2000

BIOASSAY FOR THYROID STIMULATING ANTIBODIES

The present invention relates to an assay for measuring antibodies to the thyrotropin receptor; to an assay kit therefor; and specifically to the use therein of a cell line transfected with a-luciferase cDNA.

The thyrotropin receptor (hereinafter 'TSH-R') is known to regulate both the function and proliferation of the thyroid cell, and is stimulated by the hormone thyrotropin (TSH). The TSH-R is also a target for autoantibodies, which inhibit the binding of TSH to the receptor. These autoantibodies either block the action of TSH (TBAb) (*i.e.* TSH antagonists, which act as hypostimulants or inhibitors) or stimulate (hyperstimulate) the thyroid (TSAb) by acting as agonists to TSH.

Stimulation by TSAb is believed to be a mechanism operating in Graves' disease (GD), whilst inhibition by TBAb is believed to be the case in idiopathic myxoedema. Patients with hyperthyroid Graves' disease produce antibodies which mimic the action of TSH, leading to chronic stimulation of adenylate cyclase; and whereas the autoantibodies in some patients with idiopathic myxoedema are also able to bind to the TSH-R, nevertheless this does not result in an increase in intracellular cyclic adenosine 3',5'-monophosphate (cAMP).

Many assays have been developed to measure TSH-R autoantibodies. The most widely used is a radioreceptor assay in which the binding of bovine ^{125}I-TSH to detergent-solubilised porcine TSH-R is inhibited by immunoglobulins or sera from patients suspected of having TSH-R autoantibodies. The main problems with this assay are that it uses a non-human antigen; and that it measures binding and not biological activity, hence it is not able to distinguish between TSAb and TBAb.

It is clearly desirable to be able to distinguish between stimulation and inhibition, and therefore attempts have been made to develop a bioassay in which an effect of TSH (or its inhibition by TBAb) or TSAb is measured, such as the increase in cAMP. This may be done in thyroid slices or thyroid cells in

culture and the greatest sensitivity, defined as the highest percentage of Graves' patients being positive, is achieved when the assay is performed in hypotonic (sodium chloride-free) medium. Both the radioreceptor and the bioassays are hampered by limited availability of biological material. To overcome this, a rat thyroid cell line (FRTL-5) has been developed, but here species differences may still be problematic. The recent cloning and sequencing of the TSH-R give unlimited access to recombinant human TSH-R.

As described by Libert *et al* in Biochem. & Phys. Res. Comm. 165 (3) 1250-1255 (1989) [all references herein are herein incorporated in their entirety, together with any cross-references therein], previous cloning of the dog thyrotropin receptor opened the way to molecular characterisation of the human TSH-R *via* isolation of human TSH-R cDNA clones; the analysis of the primary structure of the encoded polypeptide; and evidence that the recombinant molecule binds auto-antibodies found in patients with Graves' disease and idiopathic myxoedema. The dog TSH-R cDNA (a 2.8 kb fragment) was used to hybridise a human thyroid DNA library. Sequencing of the resulting clones gave rise to a 2292 nucleotide residue open reading frame encoding a 744 amino acid polypeptide having 90.3% similarity with the dog TSH-R. Transfection of the coding sequence in the pSVL vector of COS-7 cells allowed confirmation of the protein's ability to bind specifically TSH.

The co-transfection of Chinese hamster ovary (CHO) cells with a pSVL vector containing the coding region of human TSH-R led to the selection of cell lines particularly responsive to TSH or TSAb in terms of their cAMP accumulation (reported by Perret *et al* in Biochem. & Biophys. Res. Comm. 171 (3) 1044-1050 (1990)). Dose response curves of TSH-mediated cAMP accumulation were reported for clones JP14, JP26 and JP28, and the number of receptors per cell were found to be highest in clones JP14 and JP09.

This work gave rise to the possibility of a bioassay in which cAMP production is measured in CHO cells stably transfected with the human TSH-

R in the presence of an autoantibody either alone (TSAb) or in the presence of TSH (TBAb), (Ludgate *et al* in Molec. & Cell. Endocrin. 73 R13-R18). However, such an assay is not sufficiently robust for routine use since it takes several days to perform, especially in view of the final detection of the generated cAMP by RIA, and requires tissue culture facilities.

Accordingly, the present invention provides an assay method for TSH-R autoantibodies or TSH comprising step:

(a) contacting a test sample, in the presence or absence of TSH, with cells from a clone expressing human TSH-R transfected with a reporter construct comprising cDNA of both a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein, and a promoter containing cyclic AMP (cAMP) response elements (CREs), whereby cAMP levels vary with expression of the reactant.

Preferably, the assay method further comprises step:

(b) adding the corresponding substrate to cells thus contacted.

More preferably, the assay method still further comprises steps:

(c) measuring the response in the cells exposed to the substrate; and

(d) comparing the response from test step (c) with the response from a standard or normal sample which has undergone steps (a) to (c).

The present invention therefore especially provides an assay, for TSH-R auto-antibodies or TSH, comprising:

(a) bringing into contact a test sample with cells from a clone expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both a reactant capable of causing a measurable response when brought into contact with a corresponding substrate and a promoter containing cAMP

response elements whereby cAMP levels vary with expression of the reactant;

(b) adding the corresponding substrate to cells thus contacted;

(c) measuring the response in the cells exposed to substrate; and

(d) comparing the response from test step (c) with the response from a standard or normal sample which has undergone steps (a) to (c).

In the assay method of the present invention, all reagents used therein may be brought together in one or more steps, such as steps (a) to (d) defined herein. The notation of the steps (a) to (d) is not to be construed as meaning only that each step is carried out sequentially or that each component in the assay must be brought into individual contact with each other. For example, two or more of the steps (a) to (d) may be carried out substantially simultaneously; and/or all reagents used therein may be brought together in one step. The assay method or any combination of the steps therein may be carried out by manual, partly automated or fully automated means.

Suitable reporter constructs (referred to in step (a)) are those in which the enzyme activity results in a colour change, fluorescence change or emission of light. Examples of such enzymes include chloramphenicol acetyl transferase (CAT), Firefly luciferase, Renilla luciferase, ß-galactosidase, alkaline phosphatase, horseradish peroxidase or green fluorescent protein.

The cyclic AMP response element (CRE) of the reporter gene could be any promoter sequence or synthetic oligonucleotide which contains the CRE consensus sequence, TGACGTCA, preferably as a number of tandem repeats. A suitable promoter is that for the glycoprotein hormone alpha subunit which contains tandem cAMP response elements, described by Kay *et al* in Endocrinology 134 (2) 568-573 (1994). Another example is a construct driving the CAT enzyme which has been described by Chatterjee *et al* in Molecular Endocrinology 5 (1) 100-109 (1991).

However, the assay according to the present invention preferably comprises, in step (a), the use of a luciferase cDNA driven by a promoter containing cAMP response elements; and, in step (b), the use of luciferin; which means that the response measured in steps (c) and (d) is light output from the luciferinised cells. Preferably, the luciferase is Firefly luciferase, although Renilla luciferase or the like would also be suitable.

Most preferably, the reporter construct is α-luciferase, being a luciferase cDNA driven by the promoter for the glycoprotein hormone α subunit, mentioned above. For example, the plasmid pA3luc may be employed, having the glycoprotein hormone α subunit promoter introduced as described by Maxwell *et al* in Biotechniques 7 276-80 (1989). The α-luciferase is therefore 846 base pairs of 5' flanking sequence and 44 base pairs of exon 1 of the glycoprotein hormone α subunit promoter in the plasmid pA3luc. Alternatively, the CRE-containing sequence could be sub-cloned into a commercially-available luciferase reporter system such as the pGEM-luc vector from Promega. A further alternative is to use a plurality of plasmids, such as in the system available from Stratagene (CREB reporting system, no. 219010), which includes plasmids enabling a luciferase response to be measured following an increase in cAMP. Alternatively, inducible plasmids other than CREs could be included.

The cells may be obtained as described in the reference mentioned above by Perret *et al*. Alternatively, the human TSH-R could be subcloned into any eukaryotic expression vector (of which pSVL is an example) available from Stratagene, InVitrogen or the like for transfection into any eukaryotic cell or cell line. For selectivity, the more recently-developed dual vectors that incorporate the antibiotic resistance gene within the same plasmid, such as pcDNAIII (available from InVitrogen) may be used. Otherwise, a separate plasmid for selection may be employed.

Preferably, the cells used in the assay (step (a)) are those identified as from clone JP09 in the above-mentioned reference which have been stably

transfected with (*ie* which express) in the order of 10^5 human TSH-R per cell. More preferably, they are co-transfected with both α-luciferase cDNA and a puromycin resistance encoding plasmid such as pSV_2Neo (available from Clontech) to allow selection of assay cells with puromycin. Surviving cells are then tested for luciferase activity in response to TSH. Alternatively, cells which have been transiently transfected with any of these plasmids may be employed.

Therefore, the present invention provides a bioassay comprising human TSH-R expressed in, for example, CHO cells, wherein the improvement comprises (in place of an RIA for cAMP) measuring light output from a luciferase gene driven by a promoter containing CREs. This makes the assay more rapid, enabling the complete evaluation of TSAb, from the point of serum being in contact with the cells through to obtaining data for calculation, within a single working day. Furthermore, this assay can be performed on unfractionated serum, eliminating the need for sample preparation.

Preferably, the cells would be lyophilised (freeze-dried), frozen or comprised in a gel and provided in individual containers with one container being used per assay. Alternatively, the cells could be frozen or incorporated into a gel (such as Matrigel ™), for storage.

Another co-transfection may be carried out to provide the assay with a method of correcting for the number of cells seeded in a well during use, in the case where non-lyophilised cells are to be used. Since the Renilla luciferase construct is constitutive and has different substrate requirements from Firefly luciferase, it provides such a method. The same value would be expected from every well whilst differences would reflect varying cell number. An appropriate plasmid for this transfection is the Renilla luciferase plasmid available from Promega, no. E2241. It contains the Herpes simplex virus thymidine kinase promoter upstream from Renilla luciferase, which is thus constitutively expressed.

Furthermore, when using intact rather than lyophilised cells, in order to prevent distortion of the assay by the presence of any TSH present in serum used in the cell culture medium, the serum should be charcoal-stripped at around 24 hours prior to assay.

In addition, as with the RIA, TSH responsiveness is reduced in salt-free (*ie* NaCl-free) conditions. To further enhance assay sensitivity, reagents such as phosphodiesterase inhibitors may be added.

The present invention therefore further provides a reporter construct comprising cDNA of both a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate and a promoter containing cAMP response elements, whereby cAMP levels vary with expression of the substrate, in particular wherein the reactant is a luciferase.

Accordingly, the present invention further provides cells from a clone expressing human TSH-R (preferably,stably) transfected with a reporter construct comprising cDNA of both a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein, and a promoter containing CRE; the clone; cDNA or mRNA expressing the (preferably stably) transfected human TSH-R; and human TSH-R (preferably stably) transfected with a reporter construct comprising cDNA of both a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate and a promoter containing CRE.

Preferably, the assay according to this invention is carried out by means of a kit to enable fast and convenient results in a regular medicinal biochemistry laboratory or hospital pathology or diagnostic laboratory. The present invention therefore further provides a kit for carrying out an assay, particularly a bioluminescent assay, of the present invention, which kit comprises:

(a) cells from a clone expressing human TSH-R transfected with a reporter construct comprising cDNA of both (i) a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein, and (ii) a promoter containing cAMP response elements whereby cAMP levels vary with expression of the reactant;

(b) a standard sample for the assay;

(c) medium for culturing and/or reconstituting the cells; and

(d) instructions for carrying out the assay according to the present invention.

The corresponding protein and reagents relating thereto, and means for carrying out the response measurements may also be provided as part of the kit. For example, the kit may further comprise:

(e) buffer for lysing the cells; and/or

(f) buffer for the reporter construct, preferably luciferase buffer; and/or

(g) corresponding substrate, preferably protein, more preferably luciferin, in buffer;

and, optionally, a luminometer.

Alternatively, for example, in the case where luciferase/luciferin are employed in the assay, a separate, commercially-available kit may be employed such as one of those available from the Promega Corporation. These commercially-available kits include no. E1483 wherein the luciferase is Firefly luciferase, and a dual-luciferase system no. E1910, which also employs Renilla luciferase.

The assay method according to the present invention and the kit therefor may be used in association with a condition or disease selected from: autoimmune thyroid disease, non-autoimmune thyroid disease, autoimmunity of non-thyroid origin and polyendocrine disease. For example, they may be used in screening patients selected from: pregnant women, those with

euthyroid eye disease, and those receiving amiodarone and/or lithium. The assay method or kit according to the invention is suitable for measuring TSAb or TBAb, or for measuring autoantibodies to the TSH-R having part of its sequence modified, such as by having one or more of its amino acids replaced or otherwise modified to include tags.

The present invention will now be illustrated with reference to the following non-limiting examples:

Example 1: Preparation of cells for use in luminescent assay for TSH and TSH-R antibodies - Use of Firefly luciferase

Chinese hamster ovary cells (CHO-K1), available from the ATCC number CCL61, were subjected to calcium phosphate co-transfection, using standard protocols (Current Protocols in Molecular Biology, 1996, John Wiley & Sons Inc. section 9.1.4), with pSV_2Neo (available from Clontech) and a eukaryotic expression vector carrying the human TSH-R gene, pSVL-hTSHR (available from G. Vassart, IRIBIIN, Brussels, Belgium), as described by Perret *et al* (1990, *ibid*). The cells were selected with 400μg/ml geneticin (G418) and cloned by limiting dilution. Clone JP09 was isolated by this method (and is also available from Prof. G. Vassart), which expresses approximately 10^5 receptors per cell, as assessed in TSH binding experiments (described by Costagliola S, Swillens S, Niccoli P, Dumont J, Vassart G, Ludgate M in J Clin Endocrinol Metab 75 1540 *et seq.* (1992)).

JP09 cells were maintained at 37C in 5% carbon dioxide in air in Ham's F12 medium with 10% foetal calf serum.

Clone JP09 was co-transfected, again using the above-noted standard calcium phosphate method, with a eukaryotic expression vector carrying the puromycin resistance gene (available from, *inter alia*, InVitrogen, Stratagene etc.) and pA3Luc (available from V. Chatterjee, Univ. Cambridge, UK). Cells were selected in puromycin, 2.5μg/ml and cloned by limiting dilution. Clones were tested for light output (see Example 3) in response to bovine TSH (from Sigma, T8931). Clones giving a good TSH response were tested with a panel

of normal human sera, the selection criterion in this case being a low light output of ≤ 1.5 relative light units.

Example 2: Preparation of cells for use in luminescent assay for TSH and TSH-R antibodies - Use of Firefly and Renilla luciferases

The method of Example 1 was followed, but after the second co-transfection involving the puromycin resistance gene, another co-transfection was carried out repeating the standard calcium phosphate method, but instead with a eukaryotic expression vector carrying the hygromycin resistance gene (available from Stratagene) and the R. luciferase plasmid E2241 (available from Promega).

Example 3: Assay for Measurement of (i) TSH bioactivity and (ii) Thyroid Stimulating (TSAB) or (iii) Blocking (TBAB) Antibodies

Culture medium for the cells was RPMI; 10% foetal calf serum (FCS); 1% glutamine; 1% pyruvate; 2% penicillin/streptomycin (and 2.5μg/ml puromycin, when amplifying cells for assay). 96 well plates were seeded with 5 x 10^4 cells prepared according to Example 1 and cultured overnight (approximately 16 hours) at 37°C in a water-saturated incubator. They were then cultured for a second night in medium containing 10% charcoal stripped calf serum instead of FCS (available from Gibco). Basal light output was measured, in triplicate wells, in the presence of 100μl of the medium containing 10% charcoal stripped serum. Standards (see below) and test samples were in the form of 10% patient serum to a final volume of 100μl of the medium containing 10% charcoal stripped calf serum. Basal, standard and test incubations were for 2-3 hours as above. The final result was expressed as relative light units (RLU) obtained by the ratio of standard:basal or test:basal.

TSH bioactivity: this test is for patients having high circulating TSH levels when measured by radio-immunoassay but low circulating free T4, indicative of hypothyroidism because of defective TSH. Standards are well-

characterised euthyroid and hypothyroid serum samples of increasing biological activity.

TSAb measurements are made in thyrotoxic patients. Standards are pooled normal human serum (having a light output of ≤1.5 RLU), bTSH and well-characterised TSAb containing sera of increasing activity.

TBAb measurements are made in the same patients as in (i), but the assay wells also contain 1mU/ml of bTSH. Standards are pooled normal sera + 1mU/ml bTSH and well-characterised TBAb containing sera of increasing activity.

Measurement of light output

Following the incubation period, supernatants were removed from the wells and the cells are washed twice in phosphate buffered saline. Light output was measured using a commercially-available kit from the Promega Corporation, no. E1483, according to the manufacturer's instructions. This involved treating the cells in lysis buffer (Promega no. E1513), adding the Firefly luciferase reagent and measuring the light output in a luminometer.

If the cells used for the assay also express Renilla luciferase constitutively (as prepared in Example 2) to give a method of standardisation of cell number/well, the Promega Dual-Luciferase system is used, no. E1910. In this case, following measurement of the Firefly luciferase as above, a reagent to quench the luminescent signal is added followed by the Renilla luciferase reagent and a second reading taken.

Example 4: Selection and Use of lulu1 Cells for Assay

JP09 cells were subjected, as described in Example 1, to standard calcium phosphate transfection, either with 5 μg cAMP-luciferase and 2 μg pBABE puro or with the puromycin resistance plasmid alone. cAMP-luciferase is 846 bp of 5′ flanking region and 44 bp of exon 1 of the glycoprotein hormone α subunit promoter, which contains two cAMP response elements (CREs) in tandem, linked to the firefly luciferase gene (as

described by Chatterjee *et al* in Mol Endocrin 5 100-110 (1991)). Pools of puromycin resistant cells were obtained following selection and tested, in 6 well plates, for light output in response to bovine TSH (as described in Example 3). Colonies were isolated using cloning rings.

(a) *Selection of lulu1*

The selected clones were cultured overnight in medium with 10% charcoal-stripped calf serum (Sigma) in place of FCS, followed by 4 hours' incubation with varying concentrations of bovine TSH. Light output was measured by luciferase reporter assay (Promega) in a Berthold luminometer. Results were calculated as the ratio of light output in the presence of TSH:light output in the absence of TSH and expressed as relative light units (RLUs). Clones showing a good response to TSH were then cloned by limiting dilution and re-tested with bovine and human TSH and international TSAb standard 90/672.

(b) *Determination of a Reference Range*

Approximately 2 x 10^4 lulu 1 cells were seeded in 96 well plates and switched to 100 µl/well Ham's F12 containing 10% charcoal-stripped calf serum the day before the assay. 34 euthyroid sera from individuals negative for thyroglobulin and thyroperoxidase antibodies, and having no known history of thyroid disease were tested, in duplicate, by adding 10 µl directly to the wells and incubating at 37°C for 4 hours. Cells were assayed as described in (a) above, but using a Berthold 96 well plate luminometer. Results are expressed in RLU, as the ratio between the light output in the presence of the individual serum:light output in the absence of serum. Subsequently, the 34 sera were pooled to provide a negative control.

Sera from 100 treated patients with GD, 50 negative in a commercial TBII (TSH-R) assay (TRAK, BRAHMS Diagnostica, Berlin; the cut-off was 9 units, and the functional assay sensitivity and upper limit of detection were 8 and 405 TRAK units, respectively) and 50 positive, were assayed in duplicate, by adding 10 µl serum directly to the wells. The assay was also performed on 20 Hashimoto's, 27 multinodular (8 toxic) goitre, 20 systemic lupus

erythematosus and 12 rheumatoid factor positive arthritis sera. All results were calculated in RLU as in (a) above.

(c) *Comparison of cAMP measured by luminescence/RIA*

44 of the GD sera described above, (35 TBII (TSH-R) positive) and the TSAb standard, were also assayed in a traditional bioassay in which cAMP released into the culture medium was measured by RIA. Lulu 1 were seeded in 96 well plates and the assay was performed in 100 µl/well NaCl-free Hank's medium, containing 2 mM IBMX and 10 µl individual GD or pooled euthyroid serum. cAMP was measured by the cAMP [^{3}H] assay system (Amersham) as described by Ludgate *et al* in Exp Clin Endo 100 73-4. These results are shown in the following table (*Table 1)*, in which: ^ = mean luminometer readings (n = 3) with background light emission subtracted, (SEM); and * = mean (n=2) pmoles cAMP, (SEM).

TABLE 1	^RLUs	*pmole cAMP
blank	98 (4)	
1 mU/ml TSH	1293 (93)	9.4 (0.8)
TSAb (90/672) 10mIU/ml	1245 (80)	15.0 (1.1)
Forskolin 10^{-5} M	1651 (43)	
euthyroid	127 (5)	2.2 (0.2)

Detection of TSAb in Treated GD

An upper limit of <1.45 RLU was derived from the 97.5th percentile of analysis of 34 euthyroid samples (range 0.96 - 1.48 RLU). When the GD sera were assayed in physiological conditions, 66% of the TBII negative and 80% of the TBII positive sera produced >1.5 R.L.U. in the luminescent assay, which was obtained with only 4% of the various disease group control sera.

The intra-assay variation was 11.9%, calculated using the 100 GD sera and the inter-assay variation was 14.6% calculated from the 100 GD samples measured in two separate assays by paired analysis.

33 of the sera were positive in the luminescent assay, 27 by RIA, 7 were negative in both assays and 6 were positive by RIA but negative in the luminescent assay. Results using the three assays for TSH-R antibodies in the 100 GD sera are shown in the following table (*Table 2*):

TABLE 2	TBII	TSAb (lumi)	TSAb (RIA)
All treated GD	50/100	73/100	
TBII +ve GD	50/50	40/50	
TBII -ve GD	0/50	33/50	
treated GD	35/44	33/44	27/44

In the conditions employed, the luminescent bioassay of this invention performed better than the traditional RIA measurement for cAMP, perhaps since the indirect measurement, *via* light output, amplifies the response. The behaviour of the standard exemplifies this: in the luminescent assay, the light output was approximately 10 times that of the normal pool; while, in the RIA, it gave only a 7-fold increase.

It has also been observed that TSH responsiveness is decreased in salt-free (NaCl-free) conditions, and preliminary studies show that the luminescent assay is more sensitive for measuring TSAb but less for TSH when compared with isotonic.

CLAIMS

1. An assay method for TSH-R auto-antibodies or TSH, which method includes the step (a), which is:
contacting a test sample, in the presence or absence of TSH, with cells from a clone expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both
(i) a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein, and
(ii) a promoter containing cyclic AMP (cAMP) response elements (CREs),
whereby levels of the reactant vary with induced endogenous cAMP levels.

2. An assay method according to claim 1, wherein the promoter comprises a promoter sequence or synthetic oligonucleotide which contains the CRE consensus sequence, TGACGTCA.

3. An assay method according to claim 1 or claim 2, further including the step (b), which is:
adding the corresponding substrate to cells thus contacted.

4. An assay method according to claim 3, further including the steps:
(c) measuring the response in the cells exposed to the substrate; and
(d) comparing the response from test step (c) with the response from a standard or normal sample which has undergone steps (a) to (c).

AMENDED SHEET

5. An assay method according to any preceding claim, wherein the promoter is that for the glycoprotein hormone alpha subunit that contains tandem cAMP response elements.

6. An assay method according to any of claims 1 to 4, wherein the promoter comprises a construct driving the CAT enzyme

7. An assay method according to any preceding claim, in which the measurable response is a colour change, fluorescence change or emission of light.

8. An assay method according to claim 7, wherein the reactant is selected from chloramphenicol acetyl transferase (CAT), Firefly luciferase, Renilla luciferase, β-galactosidase, alkaline phosphatase, horseradish peroxidase and green fluorescent protein.

9. An assay method according to claim 4, which comprises, in step (a), the use of a luciferase cDNA driven by a promoter containing cAMP response elements; in step (b), the use of luciferin; and, in step (c), measuring the light output from the cell lysate in the presence of luciferin.

10. An assay method according to any preceding claim, wherein the reporter construct comprises α-luciferase.

11. An assay method according to any preceding claim, wherein the clone for use in step (a) is obtainable by stable co-transfection of CHO cells or any eukaryotic cell line with a cDNA containing the coding region of hTSH-R in a eukaryotic expression vector and a cDNA containing the reporter construct comprising both the promoter and the reactant.

AMENDED SHEET

12. An assay method according to any preceding claim, wherein all reagents used therein are brought together in one or more steps; and/or wherein two or more of the steps (a) to (d) are carried out substantially simultaneously.

13. An assay method according to any preceding claim, which is carried out by manual, partly automated or fully automated means.

14. A kit for carrying out an assay according to any preceding claim.

15. A kit according to claim 14, which kit comprises:

(a) cells from a clone expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein, and a promoter containing cyclic AMP (cAMP) response elements (CREs), whereby levels of the reactant vary with induced endogenous cAMP levels;

(b) a standard or normal sample for the assay;

(c) medium for culturing and/or reconstituting the cells; and

(d) instructions for carrying out the assay.

16. A kit according to claim 15, wherein the promoter comprises a promoter sequence or synthetic oligonucleotide which contains the CRE consensus sequence, TGACGTCA.

17. A kit according to claim 15 or claim 16, further comprising:

(e) buffer for lysing the cells; and/or

(f) buffer for the reporter construct, preferably luciferase buffer;

and/or

(g) corresponding substrate, preferably luciferin, in buffer;

and, optionally, a luminometer.

18. A kit according to any of claims 14 to 17, wherein the reporter construct comprises the plasmid pA3luc having the glycoprotein hormone α subunit promoter introduced therein.

19. A kit according to any of claims 14 to 18, wherein the CRE-containing sequence is sub-cloned into a commercially-available luciferase reporter system, such as pGEM-luc.

20. A kit according to any of claims 14 to 18, wherein the reporter construct comprises a plurality of plasmids.

21. A kit according to any of claims 14 to 18, wherein the human TSH-R is sub-cloned into a eukaryotic expression vector.

22. A kit according to claim 21, wherein said eukaryotic expression vector is pSVL.

23. A kit according to claim 21, wherein the TSH-R is sub-cloned into a dual vector that incorporates the antibiotic resistance gene within the same plasmid.

24. A kit according to claim 23, wherein the dual vector comprises pcDNAIII.

25. A kit according to any of claims 14 to 24, wherein the cells for component (a) are from clone JP09 as identified herein, which have

been stably transfected with, in the order of, 10^5 TSH-R per cell.

26. A kit according to claim 25, wherein said cells are co-transfected with both α-luciferase cDNA and a puromycin resistance encoding plasmid.

27. A kit according to any of claims 14 to 26, wherein the cells are lyophilised (freeze-dried), frozen or comprised in a gel, and provided in individual containers.

28. A kit according to any of claims 14 to 26, wherein said cells are further co-transfected to provide the assay with a method of correcting for the number of cells seeded in a well during use.

29. A kit according to claim 28, wherein said cells are further co-transfected using a Renilla luciferase plasmid.

30. An assay method or a kit according to any preceding claim for use in association with a condition or disease selected from: autoimmune thyroid disease, non-autoimmune thyroid disease, autoimmunity of non-thyroid origin and polyendocrine disease.

31. An assay method or a kit according to any preceding claim for use in screening patients selected from: pregnant women, those with euthyroid eye disease, and those receiving amiodarone and/or lithium.

32. An assay method or kit according to any preceding claim for measuring TSAb or TBAb, or for measuring auto-antibodies to the TSH-R having part of its sequence modified, such as by having one or more of its amino acids replaced or otherwise modified to include tags.

33. Use of a reporter construct comprising cDNA of both
(i) a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein
and
(ii) a promoter containing cAMP response elements (CREs),
whereby levels of the reactant vary with induced endogenous cAMP levels, which use is in an assay method or in the preparation of a kit, characterised in that said assay or kit is as defined in any preceding claim.

34. A use according to claim 33, wherein the promoter comprises a promoter sequence or synthetic oligonucleotide which contains the CRE consensus sequence, TGACGTCA.

35. A use according to claim 33 or claim 34, wherein the reactant enzyme is a luciferase and/or the substrate is luciferin.

36. A clone expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both
(i) a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein
and
(ii) a promoter containing cAMP response elements (CREs),
whereby levels of the reactant vary with induced endogenous cAMP levels.

37. A clone according to claim 36, wherein the promoter comprises a promoter sequence or synthetic oligonucleotide which contains the

CRE consensus sequence, TGACGTCA.

38. A clone according to claim 36 or claim 37, wherein the reactant enzyme is a luciferase and/or the substrate is luciferin.

39. Cells produced by a clone according to any of claims 36 to 38.

40. cDNA or mRNA expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both
(i) a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein
and
(ii) a promoter containing cAMP response elements (CREs),
whereby levels of the reactant vary with induced endogenous cAMP levels.

41. cDNA or mRNA according to claim 40, wherein the promoter comprises a promoter sequence or synthetic oligonucleotide which contains the CRE consensus sequence, TGACGTCA.

42. cDNA or mRNA according to claim 40 or claim 41, wherein the reactant enzyme is a luciferase and/or the substrate is luciferin.

43. Human TSH-R stably transfected with a reporter construct comprising cDNA of both a reactant, such as an enzyme, capable of causing a measurable response when brought into contact with a corresponding substrate, such as a protein, and a promoter containing CRE.

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BIOASSAY FOR THYROID STIMULATING ANTIBODIES

the specification of which: *(check one)*

REGULAR OR DESIGN APPLICATION

[ ] is attached hereto.

[ ] was filed on as application Serial No. and was amended on (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

[X] was described and claimed in International application No. PCT/GB98/02904 filed on 29 September 1998 and as amended on (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Country	Application Number	Date of Filing (day, month, year)	Priority Claimed
Great Britain	**9720693.2**	**30 September 1997**	**yes**

(Complete this part only if this is a continuing application.)

I hereby claim the benefit under 35 USC 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status--patented, pending, abandoned)

POWER OF ATTORNEY

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from Wynne-Jones, Laine & James as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: **Robert J. PATCH, Reg. No. 17,355, Andrew J. PATCH, Reg. No. 32,925, Robert F. HARGEST, Reg. No. 25,590, Benoît CASTEL, Reg. No. 35,041, Eric JENSEN, Reg. No. 37,855, Thomas W. PERKINS, Reg. No. 33,027, and Roland E. LONG, Jr., Reg. No. 41,949**, c/o YOUNG & THOMPSON, Second Floor, 745 South 23rd Street, Arlington, Virginia 22202.

Address all telephone calls to Young & Thompson at 703/521-2297.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: **Marian Elizabeth LUDGATE**
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